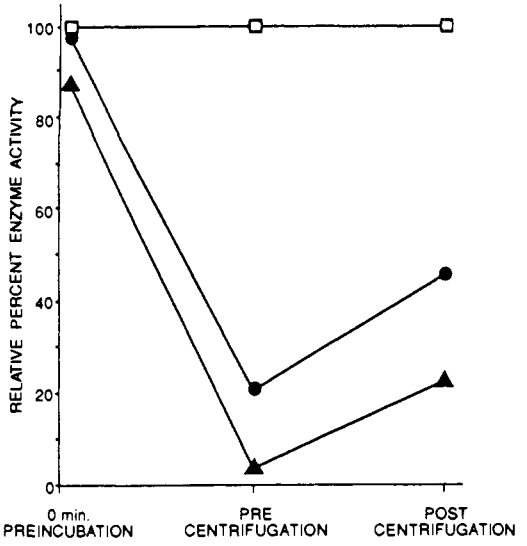

Figure 4. Reversibility of aromatase binding with 1. An NADPH-generating system was present during the 15-min preincubation period. Partial recovery of aromatase activity following isolation by centrifugation and reconstitution of human placental microsomal preparations previously treated with two concentrations of 1 (10 nM, ●; 30 nM, ▲; buffer, □). Each point represents the average of four replicates with duplicate determinations. The interassay coefficient of variation was 3.3%.

enzyme activity is statistically ($p < 0.01$) enhanced at both concentrations with the preparations containing NADPH during the preincubation periods with respect to those preparations without NADPH.

In Figure 3 are shown two sets of curves where the enzyme was preincubated with 10 and 30 nM concentrations of 1 for a fixed time (10 min), with and without NADPH. The preparations were then incubated in the presence of 500 nM androstenedione and added NADPH for 60 min with periodically monitored enzyme activity. At the early incubation timepoints the preparations with NADPH in the preincubation phase showed a greater amount of enzyme inhibition. At the 60-min assay timepoint, the effects of preincubating the enzyme and inhibitor without NADPH were not evident, as expected, since the cofactor was present during the incubation phase. We conclude from these studies that a statistically significant ($p < 0.01$) portion of the aromatase inhibition displayed by bridged steroid 1 is attributed to a cofactor-dependent process.

To further define the mechanism of inhibition of 1 we performed a reversibility assay. Two concentrations of 1 were incubated with human placental microsomes and aromatase activity was assayed as shown in Figure 4. The inhibited microsomes were isolated by centrifugation and the microsomal pellets were rinsed with assay buffer, resuspended in the assay medium, and assayed for percent relative enzyme activity. A portion of the inhibitor was removed during the centrifugation-rinsing process and a portion remained enzyme bound, as shown by the partial recovery of enzyme activity. For a strictly competitive inhibitor which did not exhibit time-dependency, we have shown a complete removal of inhibitor from the microsomes by centrifugation and rinsing. Also, for an inhibitor which irreversibly binds to aromatase, we have shown that the enzyme remained inactivated following the centrifugation-rinsing process.[18]

In summary, we have shown that A-ring bridged steroid 1 is a potent, time-dependent, and active site directed inhibitor of human placental aromatase. The inhibition appears to be mainly a tight-binding competitive process. However, a portion of the inhibitory activity is NADPH dependent and is consistent with the mechanism-based oxidative process proposed for this compound. It is recognized that other factors may be responsible for the NADPH-dependent behavior of 1, such as a conformational change in the enzyme active site induced by NADPH binding, or an NADPH-mediated oxidation of 1 to give a higher affinity competitive inhibitor.

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(18) We have recently described this assay. See: Burkhart, J. P.; Weintraub, P. M.; Wright, C. L.; Johnston, J. O. Novel Silylated Steroids as Aromatase Inhibitors. *Steroids* **1985**, *45*, 357–374.

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